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A rapid and enhanced DNA detection method for crop cultivar discrimination

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ABSTRACT

In many crops species, the development of a rapid and precise cultivar discrimination system has been required for plant breeding and patent protection of plant cultivars and agricultural products. Here, we successfully evaluated strawberry cultivars via a novel method, namely, the single tag hybridization (STH) chromatographic printed array strip (PAS) using the PCR products of eight genomic regions. In a previous study, we showed that genotyping of eight genomic regions derived from *FaRE*1 retrotransposon insertion site enabled to discriminate 32 strawberry cultivars precisely, however, this method required agarose/acrylamide gel electrophoresis, thus has the difficulty for practical application. In contrast, novel DNA detection method in this study has some great advantages over standard DNA detection methods, including agarose/acrylamide gel electrophoresis, because it produces signals for DNA detection with dramatically higher sensitivity in a shorter time without any preparation or staining of a gel. Moreover, this method enables the visualization of multiplex signals simultaneously in a single reaction using several independent amplification products. We expect that this novel method will become a rapid and convenient cultivar screening assay for practical purposes, and will be widely applied to various situations, including laboratory research, and on-site inspection of plant cultivars and agricultural products.

1. Introduction

The cultivated strawberry (*Fragaria* × *ananassa*, 2*n* = 8× = 56) is one of the most economically important fruit crops in the world. Its global production in 2009 was estimated to be over 4.1 million tons (Food and Agriculture Organization of the United Nations; http://faostat3.fao.org/). Breeding programs of cultivated strawberry are conducted in many countries to improve fruit quality and yield, and to achieve extended storage capability and disease resistance. In Japan, over 30 national and/or prefectural agricultural research centers are carrying out strawberry cultivarbreeding, which has led to the creation of many popular Japanese cultivars, such as Amaou, Sagahonoka, and Hinoshizuku. These Japanese strawberry cultivars have been highly improved, which indicates that they have high productivity, earliness, and high fruit quality, including an extended shelf life. Thus, a precise and effective cultivar discrimination system is required to protect the plant proprietary right of those superior cultivars. However, discrimination based on morphological traits is affected by the environmental and/or growth conditions, and is restricted during the developmental stage (Nielsen and Lovell, 2000). Moreover, if the cultivars are closely related, it is extremely difficult to distinguish them based on morphological traits. Therefore, molecular markers have been developed based on the methods of randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and cleaved amplified polymorphic sequences (CAPS), which enable precise cultivar discrimination at any developmental stage (Arnau et al., 2001; Congiu et al., 2000; Hancock et al., 1994; Kunihisa et al., 2003, 2005; Nehra et al., 1991; Tyrka et al., 2002). However, it remains difficult to apply these methods to on-site inspection, for the following reasons: it

Abbreviations: STH, single tag hybridization; PAS, printed array strip.

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is impossible to perform cultivar discrimination within mixed and processed products, some experimental instruments are required for agarose/acrylamide gel electrophoresis, and the preparation and staining of a gel requires several hours.

Retrotransposons are ubiquitous and abundant components in virtually all known eukaryotic genomes (Feschotte et al., 2002; Feschotte and Pritham, 2007; Huang et al., 2012; Kumar and Bennetzen, 1999; Levin and Moran, 2011; Wessler, 2006). In higher plants, they usually constitute more than half of the whole genomic DNA (Bento et al., 2013; Paterson et al., 2009; Schnable et al., 2009; Tenaillon et al., 2010). They amplify the number of their copies through reverse transcription of their RNA and integration of the resulting cDNAs into new genomic loci based on the "copy-and-paste" transposition mechanism (Kumar and Bennetzen, 1999). Because of their ubiquitous distribution, high copy number, and diverse dispersion within the genome, their insertion polymorphisms among cultivars have been used as molecular markers in phylogenetic analyses, in the construction of linkage maps, and in genetic diversity studies (Flavell et al., 1998; Kalendar et al., 1999, 2011; Konovalov et al., 2010; Kumar and Hirochika, 2001; Nasri et al., 2013; Poczai et al., 2013; Smýkal et al., 2011; Syed et al., 2005). Moreover, the uniqueness of the newly integrated insertion sites has an excellent potential for the development of multiplex DNA-based marker systems that can be used to achieve cultivar discrimination. In fact, our recent research showed retrotransposon based DNA markers were useful for cultivar discrimination in several crop species, including wheat (*Triticum aestivum*) and sweet potato (*Ipomoea batatas*) (Takai and Tahara, 2011; Monden et al., 2014).

In a previous research, we successfully identified eight genomic insertion sites of the *FaRE*1 retrotransposon that were used to discriminate 32 strawberry cultivars after screening their insertion sites comprehensively (Akitake et al., 2013). *FaRE*1 has been identified as an active retrotransposon family (He et al., 2010; Melnikova et al., 2012) and shows high insertion polymorphisms even among Japanese strawberry cultivars, which are known to be genetically closely related (Akitake et al., 2013). We applied sequence-specific amplified polymorphism (S-SAP) method to investigate *FaRE*1 insertion polymorphism among 32 cultivars. This method amplifies specifically the DNA fragments between a retrotransposon end and its adjacent restriction enzyme cutting site, and visualizes multiple bands through agarose/acrylamide electrophoresis (Konovalov et al., 2010; Lou and Chen, 2007; Melnikova et al., 2012; Petit et al., 2010; Syed et al., 2005; Waugh et al., 1997). After a number of DNA fragments derived from *FaRE*1 insertion sites were cloned and sequenced, we extracted eight insertion sites by considering combinations of their polymorphisms for discriminating 32 strawberry cultivars. It was shown that the amplification of these eight insertion sites allowed the precise and rapid screening of strawberry cultivars (Akitake et al., 2013). However, this method also required agarose/acrylamide electrophoresis for signal detection, which has the difficulty in achieving on-site inspection.

In this study, we developed a novel cultivar discrimination system using the single tag hybridization (STH) chromatographic printed array strip (PAS) method, which affords the visualization of multiplex DNA signals in a single reaction with great sensitivity and in a dramatically short time. Moreover, it does not require the preparation or staining of a gel. The results of this study showed that we successfully evaluated strawberry cultivars based on the multiplex DNA signals that were derived from the amplicons of the *FaRE*1 retrotransposon and visualized using STH chromatographic PAS. Thus, we expect that this method will facilitate rapid, efficient, and highly reliable cultivar discrimination in on-site inspection of plant materials and agricultural products.

2. Materials and methods

2.1. Development of DNA markers for strawberry cultivar discrimination

This research was conducted based on the information provided from a previous research, which developed eight DNA markers for discriminating 32 strawberry cultivars (Akitake et al., 2013). Thus, we briefly described the contents of this previous research. In the previous research, 32 strawberry cultivars and its wild species (*Fragaria vesca*) were used (Supplementary Table 1). First, genomic DNA was extracted from young leaves using the DNeasy Plant mini kit (QIAGEN) following the manufacturer's protocol. After genomic DNA was digested with *Ase*I or *Rsa*I restriction enzyme (New England Biolabs Japan, Inc), forked adaptors were ligated to the digested DNA. The forked adapters were prepared by annealing two DNA oligomers: FA_AseI and FA_cmpl for *Ase*I, and FA_RsaI and FA_cmpl for *Rsa*I. PCR primers were designed based on the sequence information of *FaRE*1. We performed primary PCR with an adapter-specific (AP2) and *FaRE*1-specific (*FaRE*1_PBS) primer combination for *Ase*I digested DNA fragments, and also performed that with an AP2 and *FaRE*1-specific (*FaRE*1_LTR150_Up) primer combination for *Rsa*I digested DNA fragments. Then, nested PCR was performed with an adapter-specific (AP3) and *FaRE*1-specific (*FaRE*1_LTR_End) primer set using the initial PCR products as the template. The PCR comprised an initial denaturation at 94 °C for 2 min, which was followed by 30 cycles at 94 °C for 60 s, 75 °C for 60 s (this step was added for the amplification of *Rsa*I digested DNA fragment), 58 °C for 90 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min. PCR products were loaded on an ABI3730xl DNA Analyzer (Applied Biosystems) for DNA fragment analyses after the purification with QIA quick PCR purification kit (QIAGEN). GeneMapper software (Applied Biosystems) was used for the visualization of DNA fragment peaks. In addition, PCR products were cloned with TOPO TA cloning kit (Invitrogen), and 446 colonies were screened and sequenced. Sequences were analyzed and aligned using BLAST and ClustalW program (Larkin et al., 2007), and the sequences of 124 different *FaRE*1 insertion sites were obtained. Out of these sites, we selected eight insertion sites (Grp. 18, 41, 57, 59, 61, 65, 76 and 110) based on the combinations of their polymorphisms among 32 cultivars. The primer and adapter sequences in the previous research are listed in Supplementary Table 2.

Supplementary tables related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.06.013.

2.2. Sample preparation

We used eight strawberry cultivars (Akihime, Nyoho, Amaou, Hokowase, Benihoppe, Asuka Ruby, Red Pearl, and Kotoka) in this research. The plants of strawberry cultivars were obtained from the Tochigi Prefectural Agricultural Experiment Station and Fukuoka Agricultural Research Center. Genomic DNA was extracted from young leaves using the DNeasy plant mini kit (QIAGEN), according to the manufacturer's protocol.

2.3. PCR and signal detection by agarose gel electrophoresis

In this research, PCR was performed by amplifying the internal positive control (IPC) sequence and eight *FaRE*1 insertion sites. The IPC sequence was introduced into a pArt1 vector, which was used for the control PCR (Supplementary Fig. 1) (Mano et al., 2011). The PCR amplification for IPC sequence was carried out using PrimeSTAR GXL Taq Polymerase (Takara Bio, Ohtsu, Japan) with IPC200f and IPC200r primer combinations (Table 1) and the pArt1 vector as a template, and that for eight insertion sites was carried out using the KAPA2G Fast Mutiplex PCR kit